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Evidence for intact 5-HT_{1A} receptor-mediated feedback inhibition following sustained antidepressant treatment in a rat model of depression

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Abstract

Serotonin (5-HT) neurons are strongly implicated in mood disorders such as depression and are importantly regulated by feedback inhibition mediated by 5-HT_{1A} receptors. These receptors may play a role, albeit a poorly understood one, in the generation of mood disorders, treatment response to antidepressants and delayed therapeutic efficacy. Here we sought to gain insight into the role of 5-HT_{1A} receptor-mediated feedback inhibition in these processes by studying Fos protein expression within serotonin neurons in a rat model of stress-related mood disorder, early life maternal separation (MS), combined with two-week treatment with the antidepressant fluoxetine (FLX) in adulthood. We gauged 5-HT_{1A} receptor-mediated feedback inhibition by the ability of the antagonist, WAY-100635 (WAY), to disinhibit Fos expression in 5HT neurons. We found that two-week FLX treatment dramatically inhibited Fos expression in serotonin neurons and that this effect was reversed by blocking 5-HT_{1A} receptors with WAY. Together these observations reveal that after prolonged exposure to SSRIs, endogenous 5HT_{1A} receptors continue to exert feedback inhibition of serotonin neurons. Furthermore we found unique effects of pharmacological treatments after MS in that the WAY effect was greatest in MS rats treated with FLX, a phenomenon selective to the rostral 2/3 of the dorsal raphe nucleus (B7). These results indicate that the balance between activation and feedback inhibition of serotonin neurons in B7 is altered and uniquely sensitive to FLX after early-life stress.

Keywords

Fluoxetine; Fos; raphe; serotonin; stress; swim

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1. Introduction

Selective serotonin reuptake inhibitors (SSRIs) are an important class of widely prescribed antidepressants. These drugs block the serotonin (5-hydroxytryptamine; 5-HT) transporter (SERT), which functions to clear serotonin from the extracellular space. Thus SSRIs increase the extracellular concentration of serotonin, promoting the activation of serotonin receptors. Although the pharmacological action of SSRIs to raise extracellular serotonin is fairly rapid, their clinical efficacy often requires prolonged use. This delay in clinical efficacy is an important limitation to effective treatment and a problem that has proven difficult to overcome.

While there are several potential underlying mechanisms for delayed clinical efficacy of SSRIs an appealing hypothesis has focused on the process of feedback inhibition. In particular, 5-HT_{1A} receptor-activation functions to feedback and inhibit serotonin neurons through both direct and indirect (multisynaptic) mechanisms (see review by Altieri et al 2012). By increasing the extracellular concentration of serotonin, acute SSRI treatment leads to activation of 5-HT_{1A} receptors and immediate inhibition of serotonin neurons (Fornal et al 1999). However with repeated SSRI treatment, feedback inhibition appears to desensitize and basal firing activity of serotonin neurons returns to normal (Czachura and Rasmussen 2000). Therefore the delayed therapeutic effect of SSRIs is thought to depend on the gradual desensitization of 5-HT_{1A} receptor-dependent feedback inhibition (Blier and de Montigny 1988, 1994).

Although 5-HT_{1A} receptor desensitization is an attractive explanation for delayed therapeutic efficacy of SSRIs and is supported by many basic research findings, there are some observations that have been difficult to reconcile with this hypothesis. Notably, not only do 5HT_{1A} receptors appear to desensitize after SSRI treatment, but also as a consequence of depression itself. For example, positron emission tomography studies have reported reduced 5HT_{1A} receptor binding in major depressive disorder (Drevets et al 2000, 2007; Savitz and Drevets 2013; Savitz et al 2009). In addition, several animal models relevant to depression produce a desensitization of 5-HT_{1A} receptors including uncontrollable stress (Rozeske et al 2011), chronic unpredictable stress (Bambico et al 2009), chronic mild stress (Grippio et al 2005; Lanfumey et al 1999) and sleep deprivation (Evrard et al 2006; Roman et al 2005). And finally, early life stress (neonatal maternal separation) decreased both 5-HT_{1A} receptor and SERT mRNA in the adult dorsal raphe (Bravo et al 2014). Collectively, these observations do not fit with the idea that antidepressants are initially therapeutically ineffective due to high 5-HT_{1A} receptor activity.

There are several potential explanations for such observations. One is that antidepressants could work differently in depressed vs. normal brains. In model animals, antidepressants and depression are often studied independently while in human studies their effects may be difficult to disentangle. In addition there is the possibility for regionally selective changes to feedback inhibition within the serotonin system. The dorsal and median raphe located in the preponine hindbrain provide topographically organized serotonin innervation to forebrain regions relevant for mood, motivation and affective state. Several distinct subsets of serotonin neurons within these areas can be generally grouped by common trends in

connectivity, specifically, the rostral 2/3 of the dorsal raphe (DR; B7) vs. the caudal 1/3 of the DR (B6) and the median raphe (MR; B8; reviewed by Commons 2016). An imbalance between these systems could be intrinsic to depression and indeed region-dependent changes in feedback inhibition have been suggested by studies of suicide cases (Boldrini et al 2008). Another possibility is that desensitization might not relate in a simple way to feedback inhibition, as some evidence has already suggested (Araragi et al 2013; Soiza-Reilly et al 2015).

There are few methods that allow study of active inhibitory processes that can spatially resolve activity of specific population of neurons both within and across brain nuclei. Our lab has used an approach based on disinhibition to study 5-HT_{1A} receptor-mediated control of serotonin neuron activity. Specifically, we block feedback inhibition with a 5-HT_{1A} receptor antagonist to disinhibited neurons and thus promote expression of the immediate early gene product Fos. Fos expression can be mapped within a specific cell population (serotonin neurons) both within and across multiple nuclei. This approach gives unique insight because it depends on blocking the endogenous process of feedback inhibition. While there are both direct and indirect (multisynaptic) 5-HT_{1A} receptor feedback mechanisms that could contribute to the effects, a further advantage of the current approach is in its ability to give insight into how these mechanisms summate at the level of serotonin neurons themselves. Future studies may address the separate question of how isolated 5-HT_{1A} receptor feedback mechanisms individually contribute to these net effects.

In this study we investigated 5-HT_{1A}-mediated feedback inhibition in adult rats after repeated exposure to the SSRI fluoxetine following a widely used animal model of depression: neonatal maternal separation (Vetulani 2013). Our goals included determining if SSRI effects were different in normal vs. depression-model rats and if there were region-specific changes impacting subsets of serotonin neurons. In order to examine the state of subsets of DR and MR serotonin neurons after these manipulations, we measured Fos expression specifically within serotonin neurons. To evaluate the role of 5-HT_{1A}-mediated feedback inhibition in regulating serotonin neurons, the effect of blocking these receptors was measured.

2. Methods

2.1 Experimental Design

The core experimental design involved eight experimental groups combining an early life manipulation (maternal separation/control rearing), a two-week repeated treatment in adulthood (fluoxetine/vehicle) and an acute treatment (WAY-100635/vehicle). See Figure 1A and 1B for an overview of the experimental design, group sizes, and group abbreviations. To increase the sensitivity of the Fos assay, all rats experienced a swim stress prior to tissue collection because without the swim, Fos expression in serotonin neurons is very low (Commons 2008; Soiza-Reilly et al 2015). However we did examine two additional groups of rats to determine if the effect of fluoxetine alone was dependent on the swim. These rats were control-reared and received two week repeated treatment in adulthood (fluoxetine (n=5)/vehicle (n=4)) and were similarly processed but without an acute swim stress prior to tissue collection.

2.2 Animals

A total of 84 male offspring from 20 timed-pregnant Sprague-Dawley female rats were used in this study. Pregnant females were delivered on gestation day 14 (shipped overnight), singly housed upon arrival (Charles River Laboratories; CRL:CD(SD)), and acclimated to the animal colony undisturbed until parturition. All rats used in this study were housed in triangular cages (OptiRAT; Animal Care Systems, Colorado, USA) in a light- (12:12 light:dark cycle; lights on at 7:00am), temperature- ($22^{\circ}\text{C} \pm 2^{\circ}\text{C}$), and humidity- ($35\text{--}70\% \pm 5\%$) controlled room, with food (LabDiet Prolab Isopro RMH 3000) and water available *ad libitum*. All animal procedures were reviewed and approved by the Boston Children's Hospital Institutional Animal Care and Use Committee, in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*.

2.3 Maternal separation procedure

Separation of rat pups from the dam for 3hr per day in the first two weeks of life is a widely used protocol that has been demonstrated to be effective at modeling depressive-like phenotypes (Vetulani 2013). Separation procedures vary, but typically being on the second day after birth and extend until 12 or more days after birth. In this study, we followed the procedure used by Plotsky and colleagues (2005). Briefly, one dam had a litter of only 5 pups and was excluded from the experiment; all other dams had a litter size of at least 10 pups. All dams included in the study ($N=19$) gave birth on gestation day 22 (postnatal day 0; P0). On the morning of P2, litters were culled to 12 pups each (4–9 males/litter). Half of the litters were chosen at random to undergo maternal separation. For maternally separated litters, both the dam and the litter were removed from the home cage and placed into separate cages. The dam's cage was returned to the colony, and the cage with the litter was placed on a heating pad in a quiet room adjacent to the colony. Pups were separated daily for 3hrs (start time 9:00am $\pm$ 30min) between P2–15 (14 days total). After the 3hr separation, pups were returned to the home cage (placed in nest) and then the dam was returned to the home cage. Control litters were left undisturbed except for regular cage changes and periodic weighing. All litters were weaned on P22 and housed in groups of same-sex littermates.

2.4 Repeated treatment in adulthood

All rats received 14 daily subcutaneous (s.c.) injections, beginning on P63 $\pm$ 2 days. Injections consisted of either fluoxetine hydrochloride (FLX; RTI International) 10 mg/kg/2ml in 1–2% dimethyl sulfoxide (DMSO) and 0.9% saline made fresh each day or vehicle (VEH; 2ml/kg; 1–2% DMSO in 0.9% saline). This dose of FLX was chosen based on electrophysiological evidence that the firing rate of raphe neurons has returned to baseline after the same dose and length of treatment (Czachura and Rasmussen, 2000). All injections were given s.c. rather than intraperitoneally (i.p.) to reduce any stress associated with the injection (Meijer et al 2006). Repeated injections of littermates housed together began on the same day and were divided equally and semi-randomly across drug conditions (all rats in a cage received the same drug treatment).

2.5 Acute injections, swim stress, and perfusion

One day after the last (14th) repeated treatment injection (P77 $\pm$ 2 days), all rats were transported from the colony to a behavioral testing room. No more than 2 rats from each litter were used in any treatment condition. Rats first received an acute injection of either WAY100635 (WAY; 0.1 mg/kg/ml in 0.9% saline, s.c.) or vehicle (0.9% saline). This dose of WAY was chosen for its activity as an antagonist of 5-HT_{1A} receptors, as demonstrated by its ability to increase the basal firing rate of DRN 5-HT neurons (Fornal et al, 1996). This dose was selected to avoid WAY effects at dopamine D4 receptors, which can occur at higher concentrations (Chemel et al 2006). Ten minutes later, rats were placed in a glass cylinder (46cm high, 20cm diameter) filled with water (26°C $\pm$ 1°C) to a depth of 30cm for 15 min. For all rats, swim occurred between 10am-2pm. After swim, rats were dried with absorbent pads and returned to their home cage. Two hours after the end of the swim (145 min after acute injection), rats were overdosed with pentobarbital i.p. (50mg/ml; 0.2ml/100g BW), and following loss of the eye blink reflex, were perfused transcardially with approximately 50ml of 0.9% saline followed by approximately 250ml 4% paraformaldehyde in 0.1M phosphate buffer (PFA; pH 7.6). After perfusion, brains were extracted and placed in PFA overnight at 4°C. The following day, a block of tissue containing the dorsal and median raphe was cut using a brain block, and the tissue was placed into 30% sucrose and 0.01% sodium azide in 0.1M phosphate-buffered saline (PBS) for several days until saturated. After freezing the brain block on dry ice, 40- μ m-thick coronal sections were cut on a freezing microtome and stored at -80°C in cryopreservative until immunohistochemical processing.

2.6 Fluorescent immunohistochemistry

Free-floating tissue sections were incubated for 4–6 days at 4°C with primary antisera containing two antibodies: 1) Sheep anti-tryptophan hydroxylase (TPH; 1:1000; Cat. # AB1541; EMD Millipore, Billerica, MA, USA), to label the rate-limiting enzyme of 5-HT synthesis, and thus 5-HT-producing neurons, and 2) Rabbit anti-Fos (Fos; 1:10000; Cat. # PC38; Calbiochem, La Jolla, CA, USA), to label the protein product of the immediate early gene *c-fos* as a general marker of neuronal activation. The TPH antibody was selected for its sensitivity for serotonin neurons, lack of nuclear labeling and species compatibility for double labeling with the Fos antibody. According to the manufacturer, the TPH antibody recognizes both TPH1 and TPH2. In unpublished experiments we determined that TPH immunolabeling is present in all neurons that had detectable serotonin immunolabeling (Immunostar Cat.# 20080; 1:1000), suggesting the false negative detection rate using the TPH antisera is extremely low. Although this TPH antibody is affinity purified, it does exhibit some cross reactivity to tyrosine hydroxylase, which is variable and more pronounced in mouse. To check if Fos expression in dopamine neurons accounted for any of the TPH-Fos-dually labeled cells, sections from rats in every group were triple labeled for tyrosine hydroxylase (Immunostar Cat.# 22941; 1:2000). However, no TPH-Fos expressing neurons in the DR or MR were also immunolabeled for tyrosine hydroxylase suggesting the false positive detection rate is also extremely low and that TPH-immunolabeled Fos neurons likely have high accuracy for serotonin neurons. Primary antisera were diluted in 0.1M PBS with 0.04% bovine serum albumin, 0.3% Triton X-100, and 0.1% sodium azide (PBSBSA-T-A). After 3 washes with PBS, tissue was incubated in secondary antisera for 90 min at room

temperature containing two fluorophores diluted in PBS-BSA-T-A: 1) AlexaFluor 488 conjugate Donkey anti-sheep (1:100; Cat. #A-11015; ThermoFisher Scientific, USA) and 2) Donkey anti-rabbit Cy3 (1:100; Jackson ImmunoResearch, West Grove, PA, USA). Sections were then rinsed in PBS, mounted onto glass slides from 0.05M phosphate buffer (containing no saline to reduce salt residue), dried for about 10 min, and cover-slipped with an aqueous mounting medium containing glycerol.

2.7 Analysis of 5-HT neuron activation- Fos and TPH double-labeling

Every fourth section throughout the extent of the dorsal and median raphe of each rat was analyzed for double-labeling of TPH and Fos. First, widefield images of both fluorophores were captured at 10x magnification using a fluorescent microscope (Olympus IX81). Using an Olympus UPlanSApo 10X/0.40 na objective, using widefield illumination the depth of field is approximately 14 microns. When sections are adhered to the slide, the z-dimension compresses in practice to about 20 microns such that most of the neurons are within or very near the focal plane. Thus confocal illumination would have modest imaging benefits while reducing the throughput rate. Then images from each rat were coded, and an experimenter blind to the rat's treatment condition conducted manual quantification of cells containing both Fos and TPH proteins from individual and merged images of the fluorophores using FIJI (ImageJ, NIH) and Photoshop (Adobe Systems). A double-labeled cell was defined as having nuclear Fos labeling completely filling the nucleus of a TPH containing neuron, to a degree that both fluorophores were easily distinguished from background. As previously described, seven sub-regions of the dorsal raphe were analyzed in addition to the median raphe (Figure 1 C-F; Commons 2008, 2016; Sperling and Commons 2011). Briefly, rostral-dorsal (RD) and rostralventral (RV) sub-regions were analyzed between -6.92 mm to -7.64 mm, mid-dorsal (MD), midventral (MV) lateral wings (LW), and median raphe (MR) sub-regions between -7.73 mm to 8.45 mm, and caudal-dorsal (CD) and caudal-ventral (CV) sub-regions between -8.54 mm and 9.26 mm; all coordinates are respective of Bregma (Paxinos and Watson 1998; Figure 1). For each sub-region, 2-3 sections were averaged to obtain one mean per region per rat. Individual means were then averaged to obtain group means. Out of a total of 84 rats, 4 were identified as outliers and excluded from analysis.

The core experimental design (Fig. 1A) was modeled as a 4-factor ANOVA with the between-subjects factors of early life experience (maternal separation/control rearing), repeated treatment (fluoxetine/vehicle), and acute test day treatment (WAY/vehicle), and the within-subject factor of region, treated as a repeated measure. Regions were either pooled into two groups based on related connectivity (as reviewed by Commons (2016): the caudal dorsal raphe (CD, CV; B6) combined with MR vs. the rostral dorsal raphe (B7; including the RD, RV, LW, MD, and MV) or handled as 8 individual sub-regions. These two approaches were taken because of the possibility that there could be very finely resolved topographic effects that could be masked by pooling data from individual subregions or, in the alternative scenario, effects could be distributed over larger regions that are related by broad patterns of connectivity. Following significant interaction effects involving region, simple effects ANOVA or Student t-tests for the involved factor or factors were performed. All graphs show means $\pm$ the standard error of the mean (SEM).

Given the multi-factorial nature of this study and the ability to make multiple pairs of comparisons, some data were repeated within the figures so that each figure would be easier to understand individually. In addition, we used dots with interconnecting lines as the graphing format to display the data from individual subregions. This format is consistent with standard displays of topographic data as the data represent a topographic pattern of Fos expression. Most importantly, this format allows for immediate appreciation of the difference between matching conditions or the delta, which is visually present in each graph as the space between the lines. This could be alternatively graphed directly as the delta or as percent change, but either of these formats has the potential to create a distorted view of the data when the baseline in each region is different, as in this data set.

3. Results

3.1 All factors interacted to influence Fos expression

When regions were pooled into two groups based on related connectivity (B7 vs. B6 + MR) there was a significant 4-way interaction between the within-subjects variable of region and the between-subjects variables of early life experience, repeated treatment, and acute treatment ($F_{1,72}=4.00$, $p<0.05$). When regions were broken down into 8 individual subregions the 4-way interaction term was no longer significant ($F_{7,504}=1.22$, $p=0.29$), although there was a significant 3-way interaction between region, repeated treatment and acute treatment ($F_{7,504}=12.63$, $p<0.05$). Given these multiple interactions, we begin by describing the pharmacological effects focusing on control-reared rats, then move to the effect of maternal separation.

3.2 Fluoxetine decreases 5-HT neuron Fos

Figure 2 illustrates the effect of fluoxetine in rats with control rearing (CON-VEH vs. CON-FLX). Fluoxetine treatment robustly reduced Fos expression within TPH immunolabeled neurons (main effect of fluoxetine: $F_{1,72}=31.71$, $p<0.05$). The effect of fluoxetine was evident in every subregion, regardless of whether regions were pooled (Fig. 2A) or analyzed individually (Fig. 2B; $p's<0.05$). Representative images from a vehicle-treated and fluoxetine-treated rat are depicted in Fig. 2C. Since previous studies have shown recovery of baseline pacemaker firing rate in raphe neurons after two-weeks of fluoxetine treatment (Czachura and Rasmussen 2000) this result was unexpected. Additional control-reared groups of rats were examined to determine if these effects were dependent on the swim stimulus. In rats not subjected to swim, fluoxetine also significantly decreased Fos expression, albeit from a lower baseline ($F_{1,7}=7.45$, $p<0.05$) and the effect of fluoxetine was similarly independent of region ($F_{1,49}=0.94$, $p>0.05$; data not shown).

3.3 A 5-HT_{1A} receptor antagonist (WAY-100635) reverses the effect of fluoxetine on 5-HT neuron Fos

Next we explored the interaction between repeated fluoxetine treatment and 5-HT_{1A} mediated feedback inhibition in control-reared rats comparing (in Figure 3) four groups: CON-VEH, CON-FLX, CON-WAY and CON-FLX/WAY. Consistent with our previous results (Commons 2008), WAY treatment by itself increased Fos in B7 but not B6/MR (CON-VEH vs. CON-WAY). However, WAY treatment completely reversed the

ability of repeated FLX treatment to reduce Fos expression (significant interaction between WAY and FLX treatment: $F_{1,31}=10.45$, $p<0.05$; Fig. 3A).

Breaking these results down into more regional detail, WAY treatment alone (CON-VEH vs. CON-WAY) significantly increased Fos expression in 3 of the 5 B7 sub-regions of the rostral dorsal raphe (RD, RV, and LW; $p's<0.05$; Fig. 3B). However novel Fos expression was not observed in any caudal sub-region (B6 + MR; $p's>0.05$; Fig. 3B). More dramatically, WAY administration increased Fos expression in all raphe subregions of FLX-treated rats (CON-FLX vs. CON-FLX/WAY groups; $p's<0.05$; Fig. 3C). Finally, all WAY-treated rats (CON-WAY and CON-FLX/WAY groups) showed a very similar level and pattern of Fos expression ($p's>0.05$; Fig. 3D), with the exception that CON-FLX/WAY rats had significantly less Fos than CON-WAY rats in the CV subregion ($t_{15}=3.45$, $p<0.05$; Fig. 3D).

3.4 Effect of early life experience on 5-HT_{1A}-mediated feedback inhibition

Finally we examined the effects of fluoxetine and WAY in rats subjected to maternal separation (MS; Figure 4). This analysis revealed that MS rats responded differently to the pharmacological treatments, and this was particularly revealed in the WAY-treated groups and within the rostral dorsal raphe, B7 (significant 4-way interaction; $F_{1,72}=4.00$, $p<0.05$; Fig. 4A). That is, baseline levels of Fos and the ability of FLX to robustly reduce Fos were very similar between CON and MS rats. However, when WAY was combined with FLX treatment in MS rats the highest levels of Fos expression were reached (Fig. 4A). This effect was visible in B7 but not B6/MR (Fig. 4B). Comparing side-by-side the effect of WAY in CON and MS rats by region (Fig. 4C and D), the unique effects of WAY in MS appear distributed across the rostral DR, B7. Unlike in control-reared rats (Fig. 4C), MS FLX/WAY rats had significantly more novel Fos expression in all B7 subregions compared to MS WAY rats ($p's<0.05$), and similar novel Fos expression in all caudal subregions ($p's>0.05$; Fig. 4D).

4. Discussion

The current study explores adaptations in 5-HT_{1A}-mediated feedback inhibition in an animal model relevant to depression and after antidepressant treatment. There are several noteworthy aspects to the results. First, we found that two-week treatment with the SSRI fluoxetine robustly reduced Fos expression in serotonin neurons as defined by immunolabeling with TPH. The dampening effect of fluoxetine on Fos expression was reversed by the 5-HT_{1A} receptor antagonist WAY revealing substantive 5-HT_{1A} receptor-mediated feedback inhibition of raphe serotonin neurons after two weeks of SSRI treatment. Finally, maternal separation caused a shift in the balance between activation and 5-HT_{1A} receptor-mediated feedback inhibition of Fos in serotonin neurons, particularly those located in the rostral 2/3 of the DR, B7.

After two weeks of treatment, fluoxetine dramatically reduced Fos expression in serotonin neurons throughout the B7 and B6/MR nuclei. This observation was surprising given that electrophysiological evidence indicates that while fluoxetine acutely inhibits raphe serotonin neurons, after 14 days of administration pacemaker firing rate returns to normal (Czachura and Rasmussen 2000). The resolution of these contrasting results probably lies in the

limitations of the endpoints themselves. That is, pacemaker-firing rate under anesthesia may not reveal the full range of serotonin neuron function, and provides limited insight into factors such as activity dynamics and the state of second-messenger transcription dependent. On the other hand, Fos expression is at best an indirect measure of electrical activity. Our results provide evidence that serotonin neurons exist in an allostatic state after two weeks of SSRI treatment where baseline pacemaker firing rate may be normal, but there are continuing changes in transcriptional activity of Fos, which is dependent on intracellular signaling cascades (Sheng and Greenberg 1990). Consistent with this observation, Cornelisse et al (2007) suggested that changes in downstream intracellular signaling components shared by multiple neurotransmitter receptors could underlie adaptation of serotonin neurons to chronic fluoxetine treatment. This insight points to complex adaptations of raphe neurons to sustained SSRI treatment that may be important to tease out to improve understanding of the mechanism of action of SSRIs and the phenomenon of delayed therapeutic onset.

Inhibition of Fos expression due to fluoxetine treatment was reversed by blocking 5-HT_{1A} receptors with WAY. While WAY is a high affinity antagonist of 5-HT_{1A} receptors, it also has agonist activity at dopamine D₄ receptors (Chemel et al 2006). However, when given in low doses such as in this study, WAY's activity is specific to the 5-HT_{1A} receptor (Marona-Lewicka and Nichols 2011). Therefore we interpret WAY's effect here as revealing that 5-HT_{1A}-mediated feedback inhibition continues to impact raphe serotonin neurons after 14 days of SSRI treatment. This result was also unexpected because the common perception is that 5-HT_{1A} mediated feedback inhibition desensitizes after SSRI exposure, a mechanism widely thought to be relevant to the delayed therapeutic efficacy of SSRIs. It might be argued that 14 days of SSRI treatment is not sufficient to produce complete desensitization of feedback inhibition. However previous work from our lab using a similar pharmacological approach showed that even life-long increases in extracellular serotonin in serotonin transporter knockout mice (SERTKO) does not impair 5-HT_{1A} receptor-mediated feedback inhibition (Soiza-Reilly et al 2015). That is, blocking 5-HT_{1A}-mediated inhibition with WAY in the SERT-KO still had the capacity to robustly induce Fos expression in serotonin neurons. Here we show that that effect is mimicked by two-week SSRI exposure.

In this study drugs, were administered systemically. Advantages of this approach include insight into how feedback mechanisms work in sum to regulate serotonin neurons, as well as relevance to human pharmacotherapy, which is administered systemically. However it should be mentioned that retained feedback inhibition revealed by Fos expression could be mediated by multiple cellular mechanisms as there are both direct and indirect or multi-synaptic pathways of feedback inhibition (Hajos et al 1999). Interestingly each of these cellular mechanisms of feedback inhibition may adapt differently to sustained increases in extracellular serotonin (Albert et al., 2011). Therefore the current results provide a rationale for future studies to examine in more detail the participating cellular mechanisms.

The retained ability of the 5-HT_{1A} antagonist to disinhibit Fos expression is at odds with evidence of 5-HT_{1A} receptor desensitization, much of which is based on the study of 5-HT_{1A} receptor agonists (e.g. Le Poul et al 2000). More specifically, there is a shift in the slope of the dose-response curve after chronic SSRI treatment where 5-HT_{1A} receptor

agonists have reduced potency. However it has long been known that 5-HT_{1A} receptors retain some functionality, as supramaximal doses of agonists can still inhibit serotonin neurons (Czachura and Rasmussen 2000). More directly relevant to the current results, several studies have reported maintained or enhanced responses to 5-HT_{1A} *antagonists* following chronic SSRI treatment despite a reduced response to exogenous *agonists*. For example, Arborelius et al (1995) found that 5-HT_{1A} antagonists disinhibited raphe neurons after chronic exposure to citalopram. Gobbi and Blier (2001) showed that WAY dramatically increased raphe single-unit activity even after life-long elevated extracellular serotonin in the SERT-KO mouse. Also in the SERT-KO mouse, pharmacologic evidence caused Fox et al (2010) to propose maintained 5-HT_{1A}-mediated feedback inhibition. These findings are consistent with the observations of Araragi and colleagues (2013) who demonstrated a reduced response of raphe neurons to a 5HT_{1A} agonist, but found that this did not translate into impairment of 5-HT_{1A} receptor-mediated autoinhibition. Thus there are converging lines of evidence from several technical domains to indicate maintenance of 5-HT_{1A} feedback inhibition of serotonin neurons after sustained increases in extracellular 5-HT, despite reduced responses to 5-HT_{1A} receptor agonists. That is, the extent of agonist desensitization evidently does not predict measures related to feedback inhibition.

One of the important observations of the present study is that fluoxetine has unique effects on serotonin neurons in an animal model relevant to depression (maternal separation) compared to standard, control-reared rats. This difference was revealed in WAY-treated conditions, where maternally separated rats treated with fluoxetine display enhanced Fos expression across the rostral two-thirds of the dorsal raphe (B7). This finding is intriguing evidence that maternally separated rats have unique adaptations involving feedback mediated by 5-HT_{1A} receptors related to their response to SSRIs. Likewise other studies have shown persistent effects of maternal separation on relevant gene expression (5-HT_{1A} receptor and SERT) in the DR (Bravo et al 2014). Thus these observations indicate experiments utilizing normal animals might not capture unique aspects of pharmacological effects in animal models of a disorder and this constitutes an important consideration for drug discovery.

The alterations in the WAY effect after maternal separation reflect a change in a 5-HT_{1A} receptor-dependent feedback inhibitory process. However feedback inhibition is not a mechanism that functions in isolation but rather it works to counter-balance other factors that are excitatory or otherwise activating. In this case, feedback inhibition is working to balance factors that activate Fos, which are revealed when feedback inhibition is blocked. While there are no detectable differences between groups in the absence of WAY, it's difficult to rule out the possibility of changes in activating factors that oppose feedback inhibition and contribute to the WAY effect. That is, the changes caused by maternal separation may not be in feedback inhibition alone but rather we interpret the altered WAY effect more conservatively as revealing that a shift has occurred in the balance between activation and feedback inhibition.

Regional analysis of the effects of maternal separation indicated the effects specific to maternal separation are centered in B7, the rostral two-thirds of the DR. Major forebrain targets of serotonin neurons located in B7 include the extended amygdala, nucleus

accumbens and many cortical areas (Muzerelle et al 2016). Furthermore, the amygdala and cortical areas including the prefrontal cortex provide reciprocal innervation that preferentially targets B7 over B6/MR (reviewed by Commons 2016). Thus the influence of maternal separation on B7 would be consistent with the idea that dysfunction of cortical areas are intrinsic to a depressed state (Drevets et al 2008; Natarajan et al 2017). The association of B7 with cortical regions connects with lines of evidence that descending control from the medial prefrontal cortex on dorsal raphe serotonin neuron activity may be particularly important for conveying information about the controllability of stress, which modifies long-term behavioral consequences relevant to depression (Amat et al 2005).

In sum, using a combination of neuroanatomical and pharmacological methods, this study provides evidence that prolonged exposure to SSRIs shifts serotonin neurons into an allostatic state where endogenous 5-HT_{1A}-dependent feedback inhibition remains relevant. Furthermore, in the maternal separation model of early life stress, the balance of Fos activity along the rostrocaudal axis of the raphe, especially the rostral two-thirds of dorsal raphe (B7), appears altered and hypersensitive to the effects of fluoxetine. Taken together these observations point to a dissociation between evidence for 5-HT_{1A} receptor desensitization and measures of endogenous 5-HT_{1A} receptor function. Thus this work illuminates substantive gaps in our understanding of the nuances of 5-HT_{1A} receptor function and likewise casts a shadow on the most simplistic models for its' role in delayed therapeutic efficacy.

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Abbreviations:

(DR)	Dorsal raphe
(FLX)	Fluoxetine
(MS)	Maternal separation
(MR)	Median raphe
(5-HT)	Serotonin
(SERT)	Serotonin transporter
(WAY)	WAY-100635

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Highlights

- Repeated treatment with the SSRI fluoxetine inhibits 5-HT neuron-Fos
- 5-HT_{1A} receptor-mediated feedback inhibition is maintained after SSRI treatment
- Maternal separation alters feedback inhibition in the rostral dorsal raphe

B

Early Life Experience	Repeated Treatment	Test Day Treatment	Group Label	Group Size (n)
Control-Reared	Vehicle	Vehicle	CON VEH	9
	Vehicle	WAY-100635	CON WAY	7
	Fluoxetine	Vehicle	CON FLX	9
	Fluoxetine	WAY-100635	CON FLX/WAY	10
Maternal Separation	Vehicle	Vehicle	MS VEH	11
	Vehicle	WAY-100635	MS WAY	11
	Fluoxetine	Vehicle	MS FLX	12
	Fluoxetine	WAY-100635	MS FLX/WAY	11

Figure 1. Experimental design and raphe subdivisions analyzed. A) Schematic depicting experimental design. B) Group sizes and labels for all experimental conditions. C) Rostral dorsal raphe, dorsal (RD) and ventral (RV), sampled between Bregma –6.92mm to –7.64mm. Scale bar = 100 μm. D) Mid dorsal raphe, dorsal (MD), ventral (MV), and lateral wings (LW), sampled between Bregma atlas coordinates –7.73mm to –8.45mm. Scale bar = 200 μm. E) Caudal dorsal raphe, dorsal (CD) and ventral (CV), sampled between Bregma atlas coordinates –8.54mm to 9.26mm. Scale bar = 100 μm. F) Median raphe (MR) was also sampled at the

mid dorsal raphe level. Data obtained from RD, RV, MD, MV, and LW (B7) and CD, CV, and MR (B6 + MR) were pooled for analysis. Single images were stitched using FIJI (Preibisch et al (2009) *Bioinformatics* **25**: 1463–5). Scale bar = 100 μ m.

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Figure 2.

Fluoxetine (FLX) treatment reduces Fos activation within serotonin neurons. A) Two weeks of FLX treatment dramatically decreased Fos expression in serotonin neurons of the rostral dorsal raphe (B7) and in the caudal dorsal and median raphe (B6 + MR). B) Sub-regional analysis revealed that FLX robustly decreased FOS across all sub-regions. All data in A) and B) are expressed as group means $\pm$ 1 SEM, and an asterisk indicates statistical significance ($p < 0.05$) compared to vehicle-treated rats. C) Representative images depicting immunofluorescent labeling for tryptophan hydroxylase only (TPH; green), Fos only

(magenta), or both proteins overlaid (merged) from the mid-ventral (MV) subregion of the dorsal raphe of a vehicle-treated (VEH) and a fluoxetine-treated (FLX) rat. White arrows indicate double-labeled cells. Scale bar = 50 μ m.

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Figure 3.

Acutely antagonizing 5-HT_{1A} receptors reverses the effect of fluoxetine (FLX) treatment, demonstrating retained 5-HT_{1A} receptor-mediated feedback inhibition. **A)** Acute treatment with the 5-HT_{1A} receptor antagonist WAY-100635 (WAY) completely reversed the suppressing effect of repeated FLX treatment on Fos expression in serotonin neurons within B7 and B6 + MR raphe nuclei. Both with or without repeated treatment with FLX, acute WAY treatment significantly increased serotonin neuron Fos expression compared to vehicle (VEH) treatment in the rostral dorsal raphe (B7). In B6 + MR, WAY and VEH treated groups had equivalent levels of Fos, whereas WAY reversed the effect of FLX to VEH levels. * = $p < 0.05$ compared to CON VEH group. **B)** Examining these effects with more regional granularity, WAY treatment alone significantly increased Fos in serotonin neurons in the rostral (B7) raphe subregions RD, RV, and LW. * = $p < 0.05$ compared to CON VEH group. **C)** In rats treated with FLX, WAY dramatically increased Fos in serotonin neurons in all sub-regions examined, demonstrating WAY-mediated disinhibition of raphe 5-HT neurons. * = $p < 0.05$ compared to CON-FLX group. **D)** WAY treatment produced similar FOS

expression in serotonin neurons regardless of FLX treatment in almost all raphe sub-regions. In the caudal ventral sub-region (CV), WAY treatment alone induced significantly more Fos in serotonin neurons than WAY treatment in combination with FLX treatment, * = $p < 0.05$ compared to CON FLX/WAY group, although neither treatment group differed from CON-VEH in this region

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Figure 4.

Maternal separation (MS) influences 5-HT_{1A} receptor-mediated feedback inhibition in the rostral dorsal raphe (B7), but not the caudal dorsal raphe and median raphe (B6 + MR). **A**) Acutely blocking 5-HT_{1A} receptors with WAY after fluoxetine (FLX) treatment had a larger effect in the B7 of MS rats than in control-reared (CON) rats. * = $p < 0.05$ compared to VEH group. # = $p < 0.05$ compared to WAY group. **B**) In the B6 + MR raphe sub-regions, the effects of FLX and WAY were similar in CON and MS rats. * = $p < 0.05$ compared to VEH group. **C**) In control rats, WAY treatment led to similar FOS expression in all raphe regions analyzed (except for the caudal ventral; CV) regardless of FLX treatment (same data as shown in Fig. 3D). * = $p < 0.05$ compared to WAY group. **D**) In comparison, in MS rats combined FLX and WAY treatment (FLX/WAY) led to significantly higher FOS in serotonin

neurons in the rostral dorsal raphe (B7) compared to WAY treatment alone, although FOS expression was similar in these two groups in the caudal dorsal and median raphe (B6 +MR). * = $p < 0.05$ compared to WAY group.

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