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Shifting Topographic Activation and 5-HT_{1A}-Receptor Mediated Inhibition of Dorsal Raphe Serotonin Neurons Produced by Nicotine Exposure and Withdrawal

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Abstract

Nicotine activates serotonin (5-HT) neurons innervating the forebrain and this is thought to reduce anxiety. Nicotine withdrawal has also been associated with an activation of 5-HT neurotransmission, although withdrawal increases anxiety. In each case, 5-HT_{1A} receptors have been implicated in the response. To determine if there are different subgroups of 5-HT cells activated during nicotine administration and withdrawal, we mapped the appearance of Fos, a marker of neuronal activation, in 5-HT cells of the dorsal and median raphe nuclei (DR and MR). To understand the role 5-HT_{1A} receptor feedback inhibitory pathways on 5-HT cell activity during these conditions, we administered a selective 5-HT_{1A}-receptor antagonist and measured novel disinhibited Fos expression within 5-HT cells. Using these approaches, we found evidence that acute nicotine activates 5-HT neurons rostrally and in the lateral wings of the DR while there is 5-HT_{1A} dependent inhibition of cells located ventrally both at rostral and mid levels. Previous chronic nicotine exposure did not modify the pattern of Fos activation produced by acute nicotine, but increased 5-HT_{1A}-dependent inhibition of 5-HT cells in the caudal DR. This pattern was nearly reversed during nicotine withdrawal when there was evidence for caudal activation and mid- and rostral-5-HT_{1A}-dependent inhibition. These results suggest that the distinct behavioral states produced by nicotine exposure and withdrawal correlate with reciprocal rostral-caudal patterns of activation and 5-HT_{1A}-mediated inhibition of DR 5-HT neurons. The complimentary patterns of activation and inhibition suggest that 5-HT_{1A} receptors may help shape distinct topographic patterns of activation within the DR.

Keywords

rat; addiction; sensitization; inhibition; 5-HT_{1A}; feedback

Introduction

Serotonin (5-HT) neurons located in the median and dorsal raphe nuclei (MR and DR) provide the majority of 5-HT innervation to the forebrain and are associated with appetitive behavior and setting affective state (Steinbusch, 1984). Several lines of evidence suggest that forebrain 5-HT contributes to the behavioral effects of acute and chronic nicotine exposure. Nicotine increases the firing rate of neurons located in the DR and leads to a rise in extracellular 5-HT in some regions of the forebrain (Ribeiro *et al.*, 1993; Li *et al.*, 1998;

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Mihailescu *et al.*, 1998; Martinez-Gonzalez *et al.*, 2002; Mihailescu *et al.*, 2002). Activation of 5-HT neurotransmission by nicotine is known to contribute to the anxiolytic properties of acute nicotine, which are enhanced by chronic nicotine exposure (Brioni *et al.*, 1993; Olausson *et al.*, 1999; Cheeta *et al.*, 2001). In addition, 5-HT contributes to the rewarding effects of nicotine and promotes the development of nicotine addictive behavior (Carboni *et al.*, 1988).

There is also evidence that 5-HT neurotransmission is activated during nicotine withdrawal. That is, endogenous activation of several 5-HT receptors appears to contribute to nicotine withdrawal syndromes including enhanced auditory startle (Hurt *et al.*, 1997; Jorenby *et al.*, 1999; Rasmussen *et al.*, 2000), place aversion (Suzuki *et al.*, 1997), and anxiety (West *et al.*, 1991; Hilleman *et al.*, 1992; Hilleman *et al.*, 1994). Taken together, these observations suggest the paradoxical situation where 5-HT neurotransmission is both activated by exposure to nicotine and withdrawal from it, two very distinct behavioral states.

5-HT_{1A} receptors are often implicated in contributing to the response of nicotine, and their levels and function may change with chronic nicotine exposure (Benwell *et al.*, 1990; Rasmussen & Czachura, 1997; Kenny *et al.*, 2001). 5-HT_{1A} receptors are abundant in the raphe nuclei where they are located on soma and dendrites of 5-HT neurons, and thus mediate a communication between 5-HT neurons. In addition, 5-HT_{1A} receptors are located on non-5-HT neurons in the raphe as well as in other brain areas. Activation of both raphe and extra-raphe 5-HT_{1A} receptors leads to a feedback inhibition of 5-HT cell activity (Sprouse & Aghajanian, 1987; Hajos *et al.*, 1999).

The DR and MR are heterogenous nuclei and their projections to the forebrain are topographically organized. In this study we investigated the possibility that nicotine administration and withdrawal engage different subpopulations of 5-HT neurons within the DR and MR by quantifying the appearance of the immediate early gene product Fos within 5-HT neurons. In addition, we sought to understand the inhibitory influence of 5-HT_{1A} receptor-feedback mechanisms onto 5-HT neurons during these conditions. If specific subgroups of 5-HT neurons are activated under different behavioral circumstances, 5-HT_{1A}-receptor-mediated feedback inhibition may temper excitation within these same areas, or may impact different subgroups of 5-HT neurons that could have opposing functional roles. To study the role of 5-HT_{1A} mediated feedback inhibition we blocked these pathways by systemically administering a 5-HT_{1A} receptor antagonist, WAY-100635, under the premise that this may disinhibit Fos expression (Commons, 2008). That is, novel expression of Fos would indicate prior inhibition through synaptic or multisynaptic 5-HT_{1A}-receptor mechanisms.

Methods

Adult male Sprague Dawley rats (250–350 gm; Charles Rivers, Wilmington, MA) were housed four to a cage under standard conditions. Care and use of animals were approved by the Children's Hospital Institutional Animal Care and Use Committee and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For all the studies rats were accustomed to handling for 3–5 days before the experimental manipulations.

Drug Treatments

To facilitate comparisons, all the animals were implanted an osmotic minipump (Alzet, Cupertino, CA) releasing either nicotine or vehicle 7 days before acute manipulations. Minipumps were implanted s.c. between the shoulder blades in rats anesthetized with 2% isoflurane. Immediately following surgery and 24 hours later, rats were given Metacam 5

mg/kg s.c. For chronic nicotine exposure, the pump released nicotine 3.15 mg/kg/day as expressed as the base (9 mg/kg/day as the tartrate). This dose of nicotine produces plasma nicotine levels (44 ng/ml) comparable to heavy smokers (Epping-Jordan *et al.*, 1998) and a reproducible withdrawal syndrome (Epping-Jordan *et al.*, 1998; Harrison *et al.*, 2001; Malin, 2001). Control groups received minipumps releasing vehicle (saline).

On the day of the experiment, seven days after minipump implantation, rats were brought into the laboratory and given an hour to acclimate to the room and given acute drug or vehicle injections. All the acute injections, summarized in Table 1, were given s.c. and a constant volume of 1 ml/kg body weight. After acute injections rats were returned to their home cages. All rats were perfused approximately 2 hr 15 minutes after acute injections. This delay was for the drugs to take effect (15 minutes) and for Fos transcription, translation and nuclear accumulation (2 hours).

To study the acute effect of nicotine treatment rats received either: 1. No treatment 2. Acute saline injection (s.c.), or 3., Acute nicotine injection (s.c.) (Table 1A). For acute nicotine treatment, nicotine was administered at a dose of 0.4 mg/kg expressed as the base, freshly prepared from nicotine tartrate in saline and adjusted to pH 7.

In order to understand how 5-HT_{1A} receptors were acting during nicotine exposure, four additional groups were analyzed. These received chronic and/or acute nicotine followed by an acute injection of WAY-100635 (0.1 mg/kg) s.c. (Table 1B). Control groups received chronic saline and/or acute saline followed by a similar acute injection of WAY-100635.

To study nicotine withdrawal, a precipitated withdrawal model was used. For this, rats implanted with chronic saline or chronic nicotine releasing minipumps for 7 days were administered an acute dose of the neuronal nicotinic antagonist mecamylamine at 2 mg/kg s.c. on the day of the experiment, approximately 2 hrs and 15 minutes before perfusion. To study 5-HT_{1A} receptor function during withdrawal, WAY-100635 administration was delivered acutely (0.1 mg/kg s.c.) a few minutes before mecamylamine (Table 1C).

Tissue Preparation and Immunohistochemistry

For perfusion rats were injected with pentobarbital (100 mg/kg i.p.) and euthanized by perfusion of fixative through the ascending aorta once the criteria for deep anesthesia were met. Perfusate consisted of a 10 ml saline wash followed by 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer pH = 7.6. The brains were removed and a mesencephalic block containing the MR and DR was cut and left in the fixative overnight. The tissue was then equilibrated in 30% sucrose in 0.1 M phosphate buffered saline (PBS). The tissue block was frozen on dry ice and sectioned 40 microns thick using a freezing microtome. Sections were collected with a paintbrush into PBS and processed floating. For immunohistochemistry sections were incubated for 48 hours at 4°C with the primary antisera in buffers containing 0.1% bovine serum albumin (BSA), 0.3% Triton X-100 and 0.01 % sodium azide. Primary antisera included anti-tryptophan hydroxylase (TPH), the rate-limiting enzyme for 5-HT synthesis raised in sheep from Millipore (Billerica, MA, catalog number AB1541) diluted 1:2000. This antisera yields a labeling pattern definitive for the distribution of 5-HT cells. To detect Fos, Ab-5, a rabbit polyclonal antibody EMD Chemicals (Gibbstown, NJ, catalog number PC38) was used at a dilution of 1:10,000. Immunolabeling with this antisera produces characteristic nuclear staining consistent with the role of the protein in transcription (Greenberg *et al.*, 1986). Secondary antisera conjugated to the fluorophores Alexa488 (Invitrogen, Carlsbad, CA) and CY3 (Jackson ImmunoResearch, West Grove, PA) raised in donkey with minimal cross reactivity to other species were diluted 1:100 and used to detect immunolabeling. Sections were rinsed, mounted, dried, and cover-slipped with a glycerol-containing aqueous mounting medium.

Analysis of Fos—Individual rats were coded in order to conduct the analysis blind to the treatment group. Because we were specifically interested in the effect of nicotine on 5-HT neurons, the number of Fos cells that were double labeled for TPH within specific subregions of the DR and MR were quantified and compared between groups. Systematically random sampling was used and included every fourth section through the DR and MR. This yielded 3–4 non-adjacent sections from each rat for each subregion, or twice that for the lateral wings of the DR, which were sampled from both sides of each section on which they appeared. For each section, regions were digitally photographed at the same magnification such that the same area per section was sampled. By comparing single- and double-labeled images, the number of TPH cells with Fos expression was manually enumerated.

In order to evaluate if WAY-100635 effects were selective for serotonin cells, in a subset of cases we analyzed the effect of WAY-100635 on the appearance of Fos in *non*-5-HT cells in the DR. For this 4 nicotine- or saline-treatment groups, 4 rats per group, were analyzed with and without WAY-100635. These groups were chronic saline with acute saline, chronic saline with acute nicotine, chronic nicotine with acute saline, and chronic nicotine with acute nicotine. The percent increase in Fos in both TPH and non-TPH-immunolabeled cells in groups with and without WAY-100635 was calculated and summed for all regions per rat.

Subregions of the MR and DR—The DR was subdivided into the following cell groups (Fig. 1). Rostrocaudally, there were three divisions of the DR. These levels were further divided into dorsal and ventral halves by bisecting the distance between the base of the aqueduct and the ventral extreme of 5-HT neurons. At rostral levels, the midline areas are thin and no 5-HT cells are laterally displaced. Areas of analysis were rostral dorsal (RD) and rostral ventral (RV). At the mid level, the midline portions progressively widen particularly the ventral group of cells. In addition, 5-HT cells are visible in the lateral DR. At this level, subregions analyzed were: mid-dorsal (MD) mid-ventral (MV) and lateral wings (LW). The border between lateral and midline areas was distinguished by the paucity of 5-HT neurons that occurs paramedially approximately 150 microns from the midline. At caudal levels, the DR is thin and elongated with a cluster of neurons at the base of the aqueduct. Caudal subregions were: caudal dorsal (CD), caudal ventral (CV) regions. The median raphe (MR) nucleus was sampled at a mid-rostro-caudal location in sections where the lateral wings of the DR were also visible.

Statistics—Data were statistically analyzed using a multifactorial analysis of variance (ANOVA) with repeated measures (SPSS 14.0; Chicago, IL). Subregions were analyzed as the within-subject factor, while chronic and acute treatments were analyzed as between-subject factors. In the presence of significant interaction effects involving subregion, simple effects (physical slice) ANOVA for the involved factor or factors at each level of subregion was done. When the between-subject factor presented at more than two levels, Tukey's post-hoc test was performed, otherwise pairwise differences were determined using a student's *t*-test following ANOVA. Threshold for significance was $p < 0.05$. Error bars on graphs show standard error of the mean.

Results

Effect of Chronic and Acute Nicotine on Fos Activation in 5-HT Cells

First we studied the effect of chronic and acute nicotine on levels of Fos expression within 5-HT neurons (Fig. 2). Rats that had chronic nicotine or saline exposure were compared to determine the basal levels of Fos expression when there was no further acute manipulation. The effects of an acute saline injection (s.c.) were examined to determine if chronic nicotine

changed the response to this mild stressor. Finally we examined the effects of acute nicotine, and if the effect of acute nicotine was modified by prior chronic nicotine exposure. Analysis of chronic treatment (saline vs. nicotine) and acute treatment (none, saline, nicotine) over all subregions revealed a significant interaction between subregion and acute treatment ($F(14,259) = 3.558$, $p = .000$), indicating that acute treatment had region-specific effects on Fos expression. There was no significant effect of chronic nicotine treatment, such that the effects of acute nicotine were the same regardless of previous chronic nicotine exposure. By region, there was a main effect of acute treatment rostral-dorsal, rostral-ventral, mid-dorsal and in the lateral wings ($F(2, 37) = 14.521$; 10.325 ; 8.098 and 14.074 , respectively, $p < .05$). Within each of these subregions, there were no significant differences between rats that received no treatment or an acute saline injection, although acute saline treated rats tended to have higher Fos expression in 5-HT cells (Fig. 2A). However rats receiving acute nicotine treatment had significantly more Fos in 5-HT neurons compared to those receiving acute saline injections in selected subregions of the dorsal raphe (Fig. 2B, 2C). The largest effect of acute nicotine was detected in the rostral pole of the DR and 5-HT cells with Fos after acute nicotine treatment were typically located paramedially in this region (Fig. 3).

Inhibition via 5-HT1A Receptors During Nicotine Exposure

Since there was a modest effect of acute nicotine on Fos expression within 5-HT cells, and no effect of chronic nicotine, we hypothesized that neurons may be actively inhibited via 5-HT1A receptor-mediated feedback mechanisms during one or both of these conditions. In order to examine how 5-HT1A receptors-dependent processes were acting to control 5-HT neurons, we administered the 5-HT1A receptor antagonist to block receptor function. The expectation was that disinhibition could be measured as additional Fos expression (Commons, 2008). First we compared the WAY-100635 effect in rats that received chronic saline or chronic nicotine to evaluate the role 5-HT1A receptors in controlling activation of Fos within 5-HT cells. Since in these cases the rats were not challenged with a further acute manipulation, we refer to the as the 'tonic' 5-HT1A effect (Fig. 4A). Comparison of chronic treatment (saline or nicotine) and acute treatment (saline or WAY-100635) by subregion showed a significant interaction between subregion and WAY-100635 treatment ($F(7,203) = 9.690$, $p < 0.001$), indicating that WAY-100635 increased Fos in several areas (Fig. 4A). Subsequent analysis by subregion revealed significant effects of WAY-100635 in every region except ventral rostral and ventral mid (rostral dorsal ($F(1, 29) = 50.5$, $p < 0.001$), mid dorsal ($F(1,29) = 9.935$, $p = 0.004$), lateral wings ($F(1,19) = 33.252$, $p < 0.001$) caudal dorsal ($F(1, 29) = 26.607$, $p < 0.001$) caudal ventral ($F(1,29) = 31.794$, $p < 0.001$) median raphe ($F(1,29) = 22.458$, $p < 0.001$)). Pairwise comparison between the chronic saline treated rats receiving WAY-100635 vs. acute saline and separately between chronic nicotine treated rats receiving WAY-100635 vs. acute saline showed the same regions were significantly different ($p < 0.05$) except for the mid-dorsal (MD) comparison for chronic saline treated rats ($p = 0.07$).

Although the 'tonic' effect of WAY-100635 was very similar in both chronic saline and chronic nicotine treated rats, it was not identical and there was also a significant interaction between subregion and chronic treatment ($F(7,203) = 2.787$, $p = 0.009$), which occurred only in the caudal-dorsal subregion ($F(1, 29) = 7.557$, $p = .010$; Fig. 4A). Accounting for this, the WAY-100635 effect in chronic nicotine is slightly attenuated and can be distinguished from the chronic saline group ($p < 0.05$).

Acute nicotine exposure is thought to lead to an activation of 5-HT1A receptors (Mihailescu *et al.*, 2002). Therefore, we determined the WAY-100635 effect after acute nicotine and if that was modified by chronic nicotine exposure. Analysis by subregion of the effects of chronic treatment (saline or nicotine) and acute treatment (WAY-100635 alone or WAY-100635 plus nicotine) yielded a significant three-way interaction between region,

chronic and acute treatment ($F(7,189) = 5.776$, $p < 0.001$) such that previous chronic nicotine exposure changed the WAY-100635 effect during acute nicotine, and it did so in a region-specific manner (Fig 4B,C and Fig 5). In chronic saline treated rats, when WAY-100635 was administered in combination with acute nicotine, a small magnitude increase in the number of 5-HT neurons with Fos was detected rostral-ventral and mid-ventral in the DR. In these areas there was a significant effect of acute nicotine treatment ($F(1,27) = 4.633$, $p = .040$ rostral-ventral; $F(1,27) = 7.284$, $p = 0.012$ mid-ventral). In chronic nicotine treated rats exposed to acute nicotine, WAY-100635 had a robust effect on Fos expression in 5-HT cells located caudally, both dorsal and ventral. There was a significant effect of acute nicotine in the lateral wings ($F(1,27) = 6.561$, $p = 0.016$) and significant interactions between chronic and acute treatment in the caudal dorsal raphe both dorsally and ventrally ($F(1,27) = 18.097$, $p < 0.001$ and $F(1,27) = 20.649$; $p < 0.001$ respectively).

In chronic saline and chronic nicotine treated rats, with and without acute nicotine injection, the effect of WAY-100635 on both the number of 5-HT cells with Fos and non-5-HT cells with Fos was evaluated in a subset of animals ($N = 4$ per group) to determine if WAY-100635 was selective for 5-HT cells. Averaged over all areas and all groups, WAY-100635 increased the number of 5-HT cells with Fos by $160\% \pm 76\%$ (standard deviation) while there was $0\% \pm 18\%$ change in the number of non-5-HT cells with Fos.

Summarized graphically in Figure 6A and B, acute nicotine produced a modest increase in Fos expression, primarily within the rostral DR. Acute nicotine similarly activated Fos expression in the rostral DR in rats with previous chronic nicotine exposure. However, chronic nicotine changed how 5-HT_{1A} receptors are engaged to control Fos expression in 5-HT cells, particularly when the rat is acutely exposed to nicotine and this effect is selective for the caudal pole of the nucleus.

Precipitated Withdrawal

5-HT neurotransmission may also be activated during nicotine withdrawal. Therefore we examined the possibility that topographically distinct patterns of Fos activation within 5-HT cells may occur during withdrawal. Withdrawal was studied using a precipitated withdrawal model produced by injection of the neuronal nicotinic receptor antagonist mecamylamine. Analysis by subregion of the effects of chronic treatment (saline, nicotine) and acute treatment (saline, mecamylamine) resulted in a significant three way interactions between subregion, chronic nicotine and mecamylamine treatment ($F(7,161) = 3.328$, $p = 0.002$) indicating that mecamylamine had unique effects in chronic nicotine treated rats, and this was region-dependent (Fig. 7). Subsequent analysis for the effects of chronic nicotine and mecamylamine in each region revealed significant interaction effects dorsal caudal ($F(1,23) = 17.175$, $p < 0.001$) ventral caudal ($F(1,23) = 45.543$, $p < 0.001$) and mid ventral ($F(1,23) = 5.346$, $p = 0.030$).

There was no significant effect of mecamylamine in chronic saline treated rats, however there was a trend for an increase in several areas that reached borderline significance in the lateral wings ($p = 0.06$), consistent with the possibility that mecamylamine could have some effects in the absence of exogenous nicotine (Mihailescu *et al.*, 1998).

To investigate how 5-HT_{1A} receptor dependent feedback mechanisms work to control activation of Fos in 5-HT cells during withdrawal, we tested the effect of WAY-100635 in the withdrawal (mecamylamine) groups. Analysis of chronic treatment (saline or nicotine) and acute treatment (mecamylamine with or without WAY-100635) indicated that there was a significant three-way interaction between subregion, chronic nicotine and WAY-100635 exposure ($F(7,119) = 2.82$, $p = .009$). By region, mid-dorsal ($F(1,17) = 6.209$, $p = .023$) and rostral-ventral ($F(1,17) = 8.315$, $p = .010$) showed significant interaction effects, mid-ventral

was marginally significant ($F(1,17) = 2.677, p = .057$). Therefore, during withdrawal the predominate location of activated Fos expression is caudal and ventral in the dorsal raphe, while the major effects of WAY-100635 lie more rostral in the nucleus (Fig. 6).

Overall Trends

We examined if higher number of 5-HT cells with Fos in a particular region predicted a subsequent increase in Fos within the same region after WAY-100635 administration. For this analysis, the number of Fos cells in each region without WAY-100635 was plotted on the X-axis. Plotted on the Y-axis was the additional increase in Fos expression in the same condition and subregion after WAY-100635 was administered (i.e. delta increase after WAY-100635) (Fig. 9A). There was no significant correlation between these values (Pearson correlation coefficient, $r = 0.08$).

In addition, regional trends were examined by comparing the percent increase in Fos in each condition produced by WAY-100635 (Fig. 9B). This analysis magnifies effects when there is a low baseline (a low denominator for percent-change analysis), however it illustrates that there are different patterns of WAY-100635 effects under different conditions. In addition, it shows that some regions, such as the lateral wings, tend to have a high response to WAY-100635 administration, with an overall average of 240% increase. Other areas typically have smaller percent changes produced by WAY-100635, for example the rostral-ventral subregion averages a 68% increase.

Discussion

There are two noteworthy findings of this study. First, there was a difference between the effect of acute nicotine administration and nicotine withdrawal on the pattern of Fos expression within 5-HT neurons of the DR and MR. The implication of this finding is that different patterns of topographic activation of the 5-HT system could contribute to differential behavioral states produced by these manipulations. Thus, different patterns of activation could contribute to answering the paradox of how 5-HT neurotransmission can be active during both nicotine exposure and nicotine withdrawal. Second, the patterns of Fos activation and Fos disinhibition produced by blocking 5-HT_{1A} receptors during nicotine administration and withdrawal were almost reciprocally switched, that is after chronic nicotine, acute nicotine administration produced activation rostral and disinhibition caudal while nicotine withdrawal produced activation caudal and disinhibition mid and rostral. These complimentary patterns of activation and disinhibition, and the lack of correlation between the magnitude of Fos expression in the absence and presence of WAY-100635 within individual subregions, suggests that 5-HT_{1A} receptor-dependent feedback mechanisms may function to help shape topographic patterns of activation within the raphe nuclei.

Methodological Considerations

The methods used in this study are optimized for obtaining region- and cell-specific information, however there are certain considerations important for their interpretation. First, Fos expression as an indicator of activity and has well-understood limitations. That is, although Fos is expressed following biochemical activation of cells it is an indirect marker of electrical activation and thus subject to both false negative and false positive error for electrical activation. Furthermore Fos has poor temporal resolution, and likely reflects an integral of cell state over a period of time. We employed an approach of blocking 5-HT_{1A} receptors to disinhibit Fos expression to understand the endogenous inhibitory role of 5-HT_{1A} receptors (Commons, 2008). In this study we found that this manipulations appears to selectively increase Fos expression in 5-HT neurons in the raphe, rather than non-5-HT

neurons. Nevertheless, a caveat of systemic administration is that the location of the responsible 5-HT_{1A} receptors remains unknown, and they may be located either on 5-HT or non-5-HT neurons intrinsic or extrinsic to the raphe nuclei. Advantageously however, the technique yields novel insight into the net effect of 5-HT_{1A} receptor feedback mechanisms on 5-HT cells (Commons, 2008).

Acute Nicotine Effects on 5-HT Neurons

Acute nicotine activated Fos in a subset of 5-HT neurons, consistent with previous studies showing that nicotine increases the firing rate of DR neurons (Mihailescu *et al.*, 1998; Mihailescu *et al.*, 2002). However in the forebrain, previous microdialysis studies have reported that nicotine produces no change, decreases or increases in extracellular 5-HT depending on the location. Most commonly, increases in extracellular 5-HT are reported in cortical regions (Ribeiro *et al.*, 1993; Singer *et al.*, 2004; Shearman *et al.*, 2008) and the nucleus accumbens shell (Liang *et al.*, 2008; Shearman *et al.*, 2008). 5-HT neurons with Fos after acute nicotine were preferentially located in the rostral-dorsal pole of the DR, confirming our observations that this area appears to be the most reliably activated after initial exposure to nicotine (Bang & Commons, 2011). The striatum, globus pallidus and substantia nigra are preferential targets of the rostral DR (van der Kooy & Hattori, 1980; Imai *et al.*, 1986). DR 5-HT neurons projecting to the prefrontal cortex are also found in the rostral part of the DR and some of these could collateralize to the nucleus accumbens (Imai *et al.*, 1986; Van Bockstaele *et al.*, 1993). These observations taken together support the conclusion that nicotine administration leads to a topographically organized activation of 5-HT neurons in the DR potentially underlying region-specific increases in 5-HT release in the forebrain (Ribeiro *et al.*, 1993; Shearman *et al.*, 2008).

Chronic Nicotine and Adaptive Changes in the Caudal DR

Chronic exposure to nicotine had no detectable effects on the baseline expression of Fos in 5-HT cells or the activating effect of acute nicotine exposure. In addition, the 'tonic' WAY-100635 effect was very similar in both chronic saline and chronic nicotine treated rats. However, chronic nicotine exposure did produce adaptive changes within dorsal raphe networks. This was revealed by the observation that after chronic treatment, acute exposure to nicotine led to a robust 5-HT_{1A}-receptor-mediated inhibition in the caudal pole of the dorsal raphe. Interestingly, during precipitated nicotine withdrawal there was activation of Fos within 5-HT cells in similar areas. Therefore the increasing capacity of nicotine, after chronic exposure, to inhibit activation of the caudal dorsal raphe may counteract activation of these regions that occurs in nicotine withdrawal. Compared to the rostral dorsal raphe, the caudal dorsal raphe preferentially projects to the locus coeruleus and the hippocampus, areas involved in the response to stress (Imai *et al.*, 1986; Valentino & Van Bockstaele, 2008). In addition, the areas of the caudal dorsal raphe activated during withdrawal could overlap with areas activated in learned-helplessness paradigms (Grahn *et al.*, 1999) and after a swim stress (Commons, 2008), suggesting a potential correlation between activation of the caudal dorsal raphe and dysphoric and stress-related behavioral states.

Role of 5-HT_{1A} Receptors in the Dorsal Raphe

5-HT_{1A} receptors are often called the serotonin 'autoreceptor' due to their very high levels of expression within 5-HT neurons (reviewed by (Barnes & Sharp, 1999)). Furthermore, activation of 5-HT_{1A} receptors in the forebrain can function to produce a feedback-inhibition of 5-HT cells (Hajos *et al.*, 1999). In this study the effect of blocking 5-HT_{1A} receptors was typically detected in areas that did not exhibit particularly high numbers of 5-HT cells with Fos before receptors were blocked. When acute nicotine activated Fos rostrally in the dorsal raphe in chronically treated rats, the major effect of blocking 5-HT_{1A} receptors was caudal. When Fos was activated caudally in the nicotine withdrawal case, the

effect of blocking 5-HT_{1A} receptors was at mid- and rostral-levels of the DR. Moreover, we found no correlation between the effect of blocking 5-HT_{1A} receptors and the amount of Fos in a particular region prior to WAY-100635 administration. This raises the possibility that 5-HT_{1A} receptors could function in a region-non-autonomous fashion to mediate communication between different groups of neurons. Consistent with the possibility, it is known that there are many interconnections between the different raphe nuclei (Frazer & Hensler, 1999) as well as local axon collaterals of 5-HT neurons that travel for some distance within the dorsal raphe nucleus (Li *et al.*, 2001). Understanding the organization of these recurrent axon collaterals may be a future avenue of research.

The MR and different regions of the DR sampled seem to show different sensitivity to the effects of WAY-100635, with the number of 5-HT cells with Fos in lateral wings often increasing by more than two fold, while other regions typically showed more modest effects. This finding echoes subregion-selective changes in 5-HT_{1A} receptor levels observed associated with depression (Savitz *et al.*, 2009) and suicide victims (Boldrini *et al.*, 2008). Although there could be many contributing factors, one of these may be differential expression of 5-HT receptors and other molecules within in the DR (Clark *et al.*, 2006).

Conclusions

Activation produced by acute nicotine treatment is topographically distinct from the activation pattern produced by nicotine withdrawal, providing a potential resolution to the paradox of why 5-HT neurotransmission is seemingly activated during these opposing behavioral states. 5-HT_{1A} receptor-dependent inhibition of 5-HT cells in each case is primarily evident in a complementary subregional pattern. This raises the possibility that 5-HT_{1A} receptors function to help shape topographic patterns of activation within the 5-HT system, suggesting 5-HT_{1A} receptors could mediate communication between functionally different groups of 5-HT neurons.

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Figure 1.

Subregions of the DR sampled. **A.** At rostral levels (atlas coordinates from Bregma, -6.92 mm to -7.64 mm (Paxinos & Watson, 1998)) both dorsal (RD) and ventral (RV). **B.** Mid-levels (atlas coordinates -7.73 mm to -8.45 mm), dorsal (MD) ventral (MV) and bilaterally the lateral wings (LW). **C.** At the caudal pole (atlas coordinates -8.54 mm to -9.26 mm) dorsal (CD) and ventral (CV). Bar = 100 μ m.

Figure 2.

Acute nicotine treatment (0.4 mg/kg) activates Fos expression within 5-HT cells predominantly in the rostral dorsal raphe while chronic nicotine exposure (3.15 mg/kg/day for 7 days) has no significant effects. On the X-axis, regions analyzed are depicted in a rostral to caudal fashion. Y-axis is the average number of 5-HT cells containing Fos (#DL (double-labeled)) per section. **A.** Chronic nicotine or saline treated rats with no further manipulation have very few 5-HT neurons per section with detectable Fos expression. Acute saline results in a non-significant trend toward more Fos expression in 5-HT cells. **B.** Acute nicotine treatment increases in the number of 5-HT cells with Fos in a region-specific manner in chronic saline treated rats. Significant increases are detected rostrally both dorsal (DR) and ventral (VR) as well as in the lateral wings (LW). **C.** Rats exposed to chronic nicotine have statistically the same response to acute nicotine as do rats receiving chronic saline. Again the largest effect of acute nicotine is detected rostral dorsal (RD) in the dorsal raphe. Effects in other regions including RV, MD and LW were more variable and non-

significant ($p = 0.10, 0.08, 0.08$ respectively). * indicates significant effect of acute nicotine treatment, $p < 0.05$.

Figure 3.

Acute nicotine increased the appearance of Fos within 5-HT cells in the rostral dorsal raphe, particularly dorsal (RD). Immunolabeling for Fos (red in the online version) and TPH (green in the online version) in chronic saline treated rats receiving either (A.) acute saline or (B.) acute nicotine 0.4 mg/kg s.c. **A.** With acute saline injection, there are very few instances of co-existence between Fos and TPH immunolabeling (arrows) either rostral dorsal (RD) or rostral ventral (RV). **B.** Acute nicotine increases the incidence of double-labeled cells, these tended to be located paramedially (arrows). Additional non-5-HT cells with Fos are also visible within this area. **A' and B'** show the same fields illuminated only for Fos immunolabeling. All panels at same scale, Bar = 120 μ m.

Figure 4.

Effects of blocking 5-HT_{1A} receptors after chronic and/or acute nicotine. **A.** The effect of the 5-HT_{1A}-receptor antagonist WAY-100635 (0.1 mg/kg) with no further acute manipulations reflects 'tonic' 5-HT_{1A} function, and is very similar in both chronic saline and chronic nicotine treated rats, although there are two subtle differences. The first difference (a) is that in the MD subregion, WAY-100635 has a significant effect in chronic nicotine treated rats, but the effect is marginally significant in chronic saline treated rats ($p = 0.07$). The second difference (b) is that although WAY-100635 still has an effect in chronic nicotine treated rats, the effect is lower than in chronic saline treated rats ($p < 0.05$). * indicates a significant (and equivalent) effect of WAY-100635 in both chronic saline and chronic nicotine treated rats. **B. and C.** Acute nicotine engages additional 5-HT_{1A} receptors and this effect changes depending on chronic nicotine exposure. **B.** In chronic saline treated rats, acute nicotine increases the effects of WAY-100635 RV and MV. **C.** In chronic nicotine treated rats, an acute nicotine injection engages a novel WAY-100635 effect in LW, CD and CV subregions of the dorsal raphe, * $p < 0.05$.

Figure 5.

Previous chronic nicotine exposure changes the effect of WAY-100635 during acute nicotine. TPOH (green in the online version) and Fos (red in the online version) immunolabeling at the caudal ventral pole of the dorsal raphe, examples of dual-labeled cells are indicated with arrows. A'B'C'D' show the Fos immunohistochemistry by itself in the same sections. **A., B.** In the caudal ventral pole of the dorsal raphe, both chronic saline or chronic nicotine treated rats have a few 5-HT cells with detectable Fos expression after WAY-100635. **C.** After acute nicotine, chronic saline treated rats still have a few 5-HT neurons with Fos. **D.** After acute nicotine, rats that were previously exposed chronically to nicotine have robust Fos expression visible. All panels same scale, bar in D' = 50 μ m.

Figure 6.

Graphic summary of the location of 5-HT neurons where Fos was activated (red, in the online version) or disinhibited after 5-HT_{1A} receptors are blocked (blue, in the online version). Purple (in the online version) indicates when both activation and disinhibition were found in the same region. **A.** In chronic saline treated rats, acute nicotine treatment activates Fos expression rostrally, both dorsal and ventral as well as in the lateral wings of the dorsal raphe. WAY-100635 effect during acute nicotine treatment provided evidence for 5-HT_{1A} mediated inhibition controlling activity rostral-ventral (purple) and mid ventral (blue). **B.** After chronic nicotine exposure, acute nicotine activates Fos again rostral-dorsal in the dorsal raphe. However, there are unique effects of WAY-100635, particularly in the caudal pole of the dorsal raphe. **C.** During nicotine withdrawal, Fos is activated in 5-HT cells caudally both dorsal and ventral, and there is a small magnitude effect mid-ventral. During withdrawal there is evidence for 5-HT_{1A} mediated inhibition effecting the ventral rostral, mid dorsal and mid ventral components of the dorsal raphe.

Figure 7. Effect of precipitated withdrawal on activation of Fos in 5-HT cells (**A**) and disinhibition of Fos produced by WAY-100635 (**B**). **A.** When the neuronal nicotinic receptor antagonist mecamylamine (Mec, 2 mg/kg) is given to chronic saline or chronic nicotine treated rats (7 days at 3.15 mg/kg/day), there is unique activation of Fos in 5-HT cells in chronic nicotine treated rats. This occurs, caudally both dorsal and ventral along with a small magnitude effect mid-ventral. * indicates significant interaction between mecamylamine and chronic nicotine, $P < .05$. Mecamylamine does not have significant effects in chronic saline treated rats, although there is a trend for increased Fos. **B.** (Due to the magnitude of the Fos expression after WAY-100635, in this comparison the data from mecamylamine treated rats shown in panel A is re-plotted on a different scale for clarity). Administration of WAY-100635 has unique effects on rats undergoing precipitated withdrawal RV and MD. *

indicates a significant interaction between chronic nicotine/mecamylamine treatment and WAY-100635; $p < 0.05$. – indicates marginally significant interaction effect, $p = .057$.

Figure 8. Comparison of the WAY-100635 effect on Fos expression in 5-HT cells at mid levels of the dorsal raphe in withdrawal groups. **A.** Chronic saline treated rats have Fos expression visible in the lateral wings (LW, arrows), but not along the midline. **B.** Chronic nicotine treated rats have pronounced Fos expression within 5-HT cells along the midline particularly dorsal, arrows in boxed area shown at higher magnification in inset.

A. Before and After WAY-100635

B. Percent Increase After WAY-100635

Figure 9.

A. Relationship between number of 5-HT cells with Fos within each area (X-axis), and further increase in Fos after WAY-100635 administration (Y-axis) for each condition. The amount of Fos expression in the absence of WAY-100635 does not predict WAY-100635 effect within the same area: Pearson correlation coefficient $R = .08$. **B.** Percent increase in Fos expression in each area in each condition produced by WAY-100635. In different conditions, WAY-100635 produces different patterns of effects. However, some areas consistently show large effects of WAY-100635 such as the lateral wings (LW), with others tend to show a smaller effect.

Table 1

Analysis	Acute Treatment	Chronic saline	Chronic nicotine
A. Effect of acute and chronic nicotine exposure	No treatment	N=4	N=4
	Saline (1 ml/kg)	N=10	N=8
	Nicotine (0.4 mg/kg)	N=9	N=9
B. Effect of WAY-100635 during acute and chronic nicotine exposure	WAY-100635 (0.1 mg/kg)	N=8	N=8
	Nicotine + WAY-100635	N=8	N=7
C. Precipitated withdrawal and WAY-100635 effect	Mecamylamine (2 mg/kg)	N=5	N=5
	Mecamylamine + WAY-100635	N=6	N=5