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Evidence for topographically organized endogenous 5-HT-1A receptor-dependent feedback inhibition of the ascending serotonin system

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Abstract

Raphe and extra-raphe 5-HT-1A receptors contribute to feedback inhibition of serotonin (5-HT) neurons; however, the endogenous function of 5-HT-1A receptor-dependent feedback inhibition remains poorly understood. Here, the possibility that 5-HT-1A-mediated feedback inhibition of the raphe nuclei is topographically organized was examined. This was done by testing the effect of systemic blockade of 5-HT-1A receptors on Fos expression in 5-HT neurons in the dorsal raphe (DR) and median raphe (MR). The premise was that appearance of Fos after 5-HT-1A receptor blockade would implicate endogenous inhibition via 5-HT-1A-dependent processes. 5-HT-1A receptor antagonist administration (WAY-100635) in rats returned to their home cage significantly increased the number of Fos-containing 5-HT cells in the lateral wings and the ventral caudal part of the DR as compared to vehicle-injected controls, suggesting that tonic activity of brain 5-HT-1A receptors impacts on these regions. In rats receiving vehicle injections, swim, a behavior known to influence 5-HT neurotransmission, increased the number of Fos-containing 5-HT cells only in the caudal third of DR. Administration of WAY-100635 preceding a swim did not change the amount of Fos in the caudal DR, but increased the number of Fos-containing 5-HT cells in the rostral DR, lateral wings of the DR, and MR. These results confirm, using an imaging approach, that 5-HT-1A receptor-dependent feedback inhibition depends on behavioral state (return to home cage vs. swim). Moreover, they reveal that the effect of 5-HT-1A receptor blockade in each case is subregionally organized.

Keywords

Fos; raphe; rat; serotonin; stress

Introduction

A major inhibitory influence over the ascending serotonin (5-HT) system is mediated by 5-HT-1A receptors. 5-HT-1A receptors are abundant within soma and dendrites of all 5-HT neurons, where they function as inhibitory autoreceptors [reviewed by Barnes & Sharp (1999)]. In addition, 5-HT-1A receptors are located on many non-5-HT neurons that are found both within the raphe nuclei and in target areas such as the hippocampus, amygdala and frontal cortex (Chalmers & Watson, 1991; Kia *et al.*, 1996). Extra-raphe 5-HT-1A

receptors not only modulate neural activity in the forebrain, but also influence activity within the raphe through synaptic feedback loops (Hajos *et al.*, 1999). There is evidence for endogenous activation of 5-HT-1A receptors in active unconstrained cats (Fornal *et al.*, 1996). However, in less active or anesthetized subjects, 5-HT-1A receptor antagonism increases the activity of raphe neurons less consistently and less robustly (Gartside *et al.*, 1995; Fletcher *et al.*, 1996; Munday *et al.*, 1996; Lejeune & Millan, 1998; Martin *et al.*, 1999; Hajos *et al.*, 2001; Haddjeri *et al.*, 2004). These observations have left the endogenous function of 5-HT-1A-mediated feedback inhibition poorly understood.

Accumulating evidence supports a high level of topographic organization in the ascending 5-HT projections, which largely originate in the mesencephalic dorsal raphe (DR) and median raphe (MR) nuclei. Although these nuclei provide widespread innervation of the forebrain, it appears that specific subgroups of 5-HT neurons preferentially innervate functionally related areas [reviewed by Michelsen *et al.* (2007)]. Different subgroups of 5-HT neurons, particularly in the DR, can also be defined on the basis of their morphology (Steinbusch *et al.*, 1981) and colocalization with other transmitters (Commons *et al.*, 2003; Valentino & Commons, 2005; Michelsen *et al.*, 2007). Moreover, afferent innervation of the MR and subregions of the DR also shows regional differences both in source and in transmitter content (Behzadi *et al.*, 1990; Peyron *et al.*, 1998; Lee *et al.*, 2003).

Given the subregional organization of the ascending raphe, in this study we tested whether 5-HT-1A receptor-dependent feedback mechanisms may influence activity of the DR and MR in a regionally or subregionally specific manner. It is notoriously difficult to study active inhibitory processes in the brain with topographic resolution; therefore, we used a strategy employing the concept of 'disinhibition'. Specifically, we blocked the function of 5-HT-1A receptors by systemic administration of a selective antagonist, WAY-100635. If neurons are either directly or indirectly inhibited by 5-HT-1A receptor-dependent processes, blockade of that inhibition could foster biochemical activation of neurons and promote Fos expression. The effect of systemic 5-HT-1A antagonism on Fos expression was examined in rats returned to their home cage or after a behavioral paradigm known to influence activity of the ascending raphe, namely swim.

Materials and methods

Subjects

Care and use of animals was approved by the Institutional Care and Use Committee at Children's Hospital and was consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Adult male Sprague-Dawley rats (250–300 g) were housed two to a cage on a 12-h light/dark schedule. Rats were handled for at least three consecutive days before experimental procedures. Half of the rats were injected subcutaneously in the suprascapular region with saline, and the remainder were injected with WAY-100635 at 0.1 mg/kg using a solution concentration of 0.1 mg/mL in saline. Systemic injections of similar doses of WAY-100635 have peak effects in the brain within approximately 5 min that last for 1 h or more (Fornal *et al.*, 1996). Rats were returned to their home cage, and 5 min after injection, half of the rats from each injection group were assigned to the swim groups. The protocol used for swim was similar to the 'pretest swim' as used in the forced swim procedure previously described by others [reviewed by Porsolt *et al.* (1978), Detke *et al.* (1995) and Cryan *et al.* (2005)]. However, the swim paradigm used differs from the forced swim test, in that the drug administration consisted of a single acute dose, rather than subchronic administration. For the swim, rats were placed in a cylindrical glass tank (46 cm high × 20 cm diameter) filled with water (25 ± 1 °C) to a depth of 30 cm for 15 min. Immediately after the swim, rats were removed from the tank, towel dried, and returned to their home cage. Perfusion took place after a delay of 125 min after

administration of subcutaneous injections to allow for Fos expression. Experimental groups were matched, different groups were perfused on the same day, and tissue was processed in parallel. All experiments started between 10:00 and 12:00, 3–5 h after the initiation of the light cycle.

Immunohistochemistry

Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (100 mg/kg) and perfused with 500 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (4 °C, pH 7.4). Brains were removed and stored in the same fixative solution overnight (4 °C), and then equilibrated in a solution of 25% sucrose in 0.1 M phosphate buffer. Brains were frozen, and 40 µm coronal sections were cut and processed while floating.

Primary antisera were diluted in 0.1 M phosphate-buffered saline with 0.3% Triton X-100, 0.04% bovine serum albumin and 0.1% sodium azide, and incubated with the tissue for 2–3 days at 4 °C. Fos immunoreactivity was detected by incubating sections in rabbit anti-Fos sera (catalog number PC38; Oncogene Research Products, Cambridge, MA, USA) diluted 1:10 000. 5-HT neurons were detected using an antiserum raised against tryptophan hydroxylase (TPOH), the synthetic enzyme for serotonin, raised in sheep (Chemicon International, Temecula, CA, USA; 1:2000). Although this antiserum has some cross-reactivity to tyrosine hydroxylase (TH), which is present in some dopaminergic neurons in the raphe nuclei, it was selected for its very high sensitivity for TPOH. To confirm that neurons dually labeled for Fos and TPOH did not reflect false-positive identification of TH-containing neurons, several representative sections where multiple cells containing Fos and TPOH were visible were triple labeled for TH using a mouse antiserum (Chemicon International; 1:1000). Secondary antisera raised in donkey were conjugated to CY3, Alexa-488 or 7-amino-4-methylcumarin-3-acetic acid and diluted 1:200.

Data analysis

Cells dually immunolabeled with Fos and TPOH were counted in eight subregions (Fig. 1). Three rostrocaudal divisions of the DR were made: caudal was –8.54 to –9.26 mm, middle was –7.73 to –8.45 mm, and rostral was –6.92 to –7.64 mm; coordinates measured from Bregma. These three levels were then divided at the midpoint between the base of the aqueduct and the ventral extent of TPOH-containing cells. The lateral wings of the DR and the MR comprised the remaining two groups, and both were sampled in sections that also contained mid-levels of the DR. For each rat, an average of three sections was sampled, representing each of these areas. For each area, a minimum of five rats (average number of rats/group = 7) contributed to the mean number of cells containing Fos and TPOH in each group. For sampling, the relevant area was illuminated for each fluorophor and digitally photographed using conventional fluorescence microscope and a 10× objective. Dually labeled cells were manually enumerated by visualization of the individual and merged images of each fluorophor. To be considered positive for Fos, Fos immunolabeling had to completely fill the nucleus with an intensity of labeling that was easily distinguished from background levels. The mean number of dually immunolabeled cells per section was determined for each rat within a group. Group means and standard error of the means were calculated for each subregion. For each subregion, the data were analyzed using a 2×2 between-subjects factorial ANOVA with a Tukey honestly significant difference *post hoc* test with a threshold for significance of $P < 0.05$.

Results

For all treatment groups, the sections analyzed were first examined for Fos immunolabeling in other areas besides the MR and DR to confirm that Fos was detectable. Examination of

rats receiving an injection of subcutaneous saline and then returned to their home cage revealed that very few TPOH-containing neurons were dually labeled for Fos in all regions of the DR and MR (Fig. 1). Administration of WAY-100635 produced a significant increase in the number of cells dually labeled with Fos and TPOH as compared to vehicle-injected controls in the lateral wings of the DR (Figs 1 and 2), and to a lesser extent, the ventral caudal region. However, there were no significant increases in the number of cells containing Fos and TPOH cells in all other areas of the DR and MR.

In the lateral wings, as well as in the rostral dorsal DR, there was a significant interaction between WAY-100635 and swim (Figs 1–3). In both of these areas, swim potentiated the effect of WAY-100635, resulting in a dramatic increase of Fos expression in these groups of cells (Figs 1–3). At the rostral pole, dually labeled cells were most numerous dorsally, and sometimes appeared in a paramedial pattern. The number of dually labeled cells dissipated ventrally, but was still significantly different from those in the other three treatment groups in the rostral ventral group. The number of cells dually labeled with TPOH and Fos decreased more dramatically in the caudal direction, and was not statistically different from other groups at mid-rostrocaudal levels.

In both the MR and in the rostral ventral DR, there was a main effect of WAY-100635, but no significant interaction effect. In both of these cases, the only groups that showed significant differences from the saline-treated controls were those with the swim + WAY-100635 combination (Figs 1, 3 and 4). In the MR in both the swim and WAY-100635 groups, additional cells where Fos was subthreshold because immunolabeling only partially filled the nuclei at low levels of intensity were noted.

After swim, there were numerous Fos-containing cells that lacked TPOH immunolabeling (Figs 2 and 3); however, it was generally difficult to detect Fos-containing TPOH-labeled cells, consistent with the findings of our previous study (Roche *et al.*, 2003). In rats that received a vehicle injection, swim only produced a significant increase in the number of cells dually labeled with Fos and TPOH at caudal levels in the DR, both dorsally and ventrally (Figs 1 and 5). In the same areas, administration of WAY-100635 in combination with the swim did not appear to further influence Fos expression (Fig. 5).

For each region and condition where dually labeled cells were detected, tissue sections from the same rats were triple immunolabeled for Fos, TPOH and TH to determine the localization of dopamine neurons with respect to the other two markers (data not shown). TH-containing neurons were clearly detectable, and were most prominent rostrally and dorsally in the DR. A subset of TH-immunolabeled neurons contained light immunolabeling for TPOH, reflecting partial cross-reactivity of the TPOH antisera. However, no TH-containing cells were detected that were dually labeled for Fos, and nor was TH detected in cells dually labeled with TPOH and Fos.

Discussion

The results of this study provide an immunohistochemical line of evidence for tonic and state (swim)-dependent functions of endogenous 5-HT-1A receptor feedback mechanisms. 5-HT-1A antagonism revealed subregion-specific inhibitory effects of endogenous 5-HT-1A receptor-dependent mechanisms on Fos expression in the ascending raphe nuclei. These observations are consistent with the topographic organization of the ascending raphe and the possibility that 5-HT-1A feedback mechanisms serve to refine patterns of activity within the raphe nuclei.

Rather than pharmacological activation of receptors, the current methods rely on blocking endogenous activation using an endpoint that gives topographic and cell-specific

information. This approach gives unique insights into endogenous inhibitory neurotransmission, which in some respects can be more difficult to study than excitatory neurotransmission. However, the results are subject to certain methodological limitations. First, the appearance of immediate early genes such as the Fos gene indicates activation of biochemical pathways leading to protein expression, which may not be directly related to neuronal activity. Therefore, although increased Fos expression suggests that a neuron has entered a biochemically more active state, as an indicator of increased neuronal firing Fos is subject to both false-negative and false-positive error. Second, the locus of the 5-HT-1A receptors that produced our observed effects on Fos expression remains unknown. Although it may be tempting to assume that the relevant 5-HT-1A receptors are on the raphe 5-HT neurons themselves, it has been established that 5-HT-1A receptors on non-5HT neurons, both within the raphe and in other brain locations, can influence activity within the DR through synaptic or multisynaptic mechanisms (Hajos *et al.*, 1999). Indeed, 5-HT-1A receptors are also present peripherally. Therefore, the methods show the net effect of both indirect and direct effects of 5-HT-1A antagonism on 5-HT neurons.

Antagonism of 5-HT-1A receptors in rats returned to their home cage selectively increased Fos expression in 5-HT neurons in the lateral wings and to a lesser extent in the caudal ventral part of the DR. This finding implicates tonic 5-HT-1A receptor-dependent effects on the ascending raphe, which are not equivalent in all subregions examined. If activation of Fos expression were to parallel neural activation, the results could provide a possible explanation for why tonic 5-HT-1A activity is difficult to detect using *in vivo* recording methods (Gartside *et al.*, 1995; Fletcher *et al.*, 1996; Munday *et al.*, 1996; Lejeune & Millan, 1998; Martin *et al.*, 1999; Hajos *et al.*, 2001; Haddjeri *et al.*, 2004). That is, 5-HT cells in the lateral wings of the DR are sparse and more widely dispersed than in other regions of the raphe, and for *in vivo* recording, midline areas with the highest density of 5-HT neurons are typically targeted.

Antagonism of 5-HT-1A receptors in rats that experienced a swim revealed a pronounced effect on Fos expression in 5-HT neurons in the rostral DR as well as in the MR. Previous elucidation of the ascending serotonergic projection indicates that the rostral portion of the DR contributes to a dorsal ascending pathway that innervates the dorsal and ventral striatum and globus pallidus, whereas the MR provides major innervation of the septo-hippocampal system (Steinbusch *et al.*, 1981; Waselus *et al.*, 2006). Swim has been reported to increase 5-HT levels in the striatum and, although less consistently, the hippocampus (Kirby *et al.*, 1995; Adell *et al.*, 1997; Linthorst *et al.*, 2002; Umriukhin *et al.*, 2002). Therefore, blockade of 5-HT-1A-dependent feedback inhibition may reveal the underlying activation of these neurons produced by swim that otherwise eludes detection by Fos.

No effects of swim or WAY-100635 were observed at mid-rostrocaudal levels along the midline, either dorsally and ventrally. Accumulating evidence supports the model that GABAergic mechanisms triggered by corticotropin-releasing factor are engaged to inhibit certain ascending 5-HT pathways during swim (Price *et al.*, 2002; Roche *et al.*, 2003). This is consistent with decreases in 5-HT levels in the amygdala (Kirby *et al.*, 1995), a projection site of many neurons located at mid-rostrocaudal levels along the midline (Commons *et al.*, 2003).

Although there is a specific pattern of Fos expression in the rostral and mid-rostrocaudal levels visible after swim when 5-HT-1A receptors are blocked, this may simply be generated by topographically specific activation and inhibition of these regions that is produced by swim. Indeed, activation of 5-HT cells is known to be highly heterogeneous, depending on the activity of specific afferent pathways (Levine & Jacobs, 1992; Celada *et al.*, 2001; Varga *et al.*, 2003). This would be consistent with the idea that 5-HT-1A receptors function in a

region-autonomous manner, i.e. that groups of activated 5-HT neurons inhibit themselves via 5-HT-1A receptors. However, observations in the caudal raphe may suggest more complex underlying mechanisms. That is, swim by itself increased Fos expression in caudal DR 5-HT neurons, indicating that swim changes the state of neurons in this area. However, WAY-100635 did not further impact on Fos expression. This raises the possibility that 5-HT-1A-dependent mechanisms may not be strictly region-autonomous. Although this remains an open question, a similar mode of action was suggested by our previous data showing that 5-HT-1A-dependent inhibition produced by substance P is subregionally organized in the DR. That is, substance P increases the activity of cells located dorsally in the DR, while producing a 5-HT-1A-dependent inhibition of ventrally located cells (Valentino *et al.*, 2003).

In this study, we investigated the function of endogenous 5-HT-1A-dependent processes in the ascending raphe nuclei and found that the effects are dependent on behavioral state (swim vs. home cage) using a neuroanatomical endpoint. These observations confirm electrophysiology studies reporting that 5-HT-1A antagonists increase single unit activity in the DR in a state-dependent manner and are most effective during periods of heightened activity (Fornal *et al.*, 1996). Moreover, the observations suggest that 5-HT-1A-dependent effects on the ascending raphe are subregion-specific. The MR and DR provide topographically organized innervation of the forebrain, and there are region-specific increases and decreases in forebrain 5-HT levels under distinct behavioral conditions, such as swim (Kirby *et al.*, 1995). Taken together, these observations suggest that 5-HT-1A receptors contribute to tempering or refining topographically specific patterns of activity within ascending raphe projections that correlate with specific behaviors or behavioral states.

The results reveal the utility of using markers of neuronal activation combined with pharmacology and behavior to examine active inhibitory processes such as those mediated by 5-HT-1A receptors. Many questions remain regarding the endogenous function of 5-HT-1A-mediated feedback inhibition on the raphe system, including the level of regional and cellular autonomy, and plasticity in response to acute and chronic behavioral and pharmacological challenges. Investigating these parameters using the current method and others may help us to understand the neural mechanisms that underlie the wide number of behaviors that 5-HT and 5-HT-1A receptors appear to modulate.

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Abbreviations

5-HT	serotonin
DR	dorsal raphe
MR	median raphe
TH	tyrosine hydroxylase
TPOH	tryptophan hydroxylase

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Fig. 1.

Expression of Fos in tryptophan hydroxylase (TPOH)-containing cells in the eight areas of the dorsal raphe (DR) and median raphe (MR) sampled, depicted on the left. Bars depict average number of TPOH-containing cells dually labeled with Fos per section for different groups + SEM (C, control; W, WAY-100635; S, swim; S+W, swim + WAY-100635). Each area was analyzed with a 2×2 factorial ANOVA. Significant differences as compared to controls as determined using a Tukey honestly significant difference *post hoc* test using $P < 0.05$ are marked with an asterisk, and additional differences between groups are described. (1) In the dorsal part of the rostral DR, there were significant group effects for WAY-100635 ($F_{1,23} = 16.78$, $P < 0.0005$) and swim ($F_{1,23} = 9.12$, $P < 0.01$) and a significant interaction ($F_{1,23} = 9.79$, $P < 0.005$). *Post hoc* analysis showed swim + WAY-100635 to be different from each manipulation by itself. (2) In the rostral ventral DR, there were significant group effects for WAY-100635 ($F_{1,24} = 11.56$, $P < 0.003$) and swim ($F_{1,24} = 10.28$, $P < 0.004$). *Post hoc* analysis showed swim + WAY-100635 to be different from each manipulation by itself. (3) In the lateral wings, there was a significant group effect of WAY-100635 ($F_{1,25} = 21.49$, $P < 0.0002$) and a significant interaction ($F_{1,25}$, $P = 0.050$). *Post hoc* analysis showed WAY-100635 and WAY-100635 + swim to be different from the other groups as well as each other. (4, 5) At mid-levels of the DR, both dorsally and ventrally, there were no significant effects. (6) In the MR, there was a significant group effect for WAY-100635 ($F_{1,24} = 5.75$, $P < 0.03$). *Post hoc* analysis showed swim + WAY-100635 to be different from swim alone. (7) In the caudal dorsal part of the DR, there was a significant group effect of swim ($F_{1,25} = 11.3$, $P < 0.003$). *Post hoc* analysis showed swim to be different from control, and both swim and swim + WAY-100635 to be significantly different from WAY-100635 alone. (8) For caudal ventral, there were significant group effects for WAY-100635 ($F_{1,26} = 6.12$, $P < 0.03$) and swim ($F_{1,26} = 7.83$, $P < 0.01$). *Post hoc* analysis showed WAY-100635 and swim to be different from controls, but not different from swim + WAY-100635.

Fig. 2.

Fos immunolabeling (red) in tryptophan hydroxylase (TPOH)-containing (green) cells in the lateral wings of the dorsal raphe. (A) In saline-injected rats returned to their home cage, an occasional TPOH-containing cell contained detectable Fos immunolabeling (arrow). (B) TPOH-containing cells with Fos were more commonly found (some are indicated by arrows) after a systemic injection of WAY-100635 in rats returned to their home cage. (C) After swim, Fos expression increased in the lateral wings, but very few TPOH-containing cells contained Fos (arrow). (D) When WAY-100635 is administered before a swim, many TPOH cells in the lateral wings contain detectable Fos immunolabeling (some are indicated by arrows). Scale bar = 100 μ m.

Fig. 3.

Comparison of Fos expression at rostral levels of the dorsal raphe. In rats returned to their home cage, saline injection (control) (A) or WAY-100635 administration (B) yielded low levels of Fos expression in tryptophan hydroxylase (TPOH)-containing cells, although Fos could be seen in non-TPOH-containing cells (arrows). Brackets in A illustrate where the dorsal (D) and ventral (V) cells would be sampled for all panels. (C) After swim, there were numerous Fos-containing cells that tended to be more prevalent dorsally in a paramedial pattern (bracketed area), but few TPOH-containing cells had Fos. (D) WAY-100635 administration before swim promoted Fos expression in numerous TPOH-containing cells. These were most numerous dorsally, and also tended to be located paramedially (bracketed area). Bar = 100 μ m.

Fig. 4. Fos immunolabeling (red) in tryptophan hydroxylase (TPOH)-containing (green) cells in the median raphe (MR). Similar to the rostral levels of the dorsal raphe, very few MR TPOH-containing cells contained detectable Fos immunoreactivity after a saline injection (A, control), WAY-100635 administration (B), or a swim (C). However, after the combination of WAY-100635 and swim, Fos became detectable in a subpopulation of TPOH-containing neurons (arrows in D). Bar = 100 μ m.

Fig. 5.
 (A) Fos immunolabeling (red) becomes detectable in a subset of tryptophan hydroxylase (TPOH)-containing cells (green) after swim combined with saline injections only at caudal levels in the dorsal raphe (DR). (B) However, in contrast to the rostral DR and lateral wings, pretreatment with WAY-100635 before the swim had no additional effect on the number of Fos-containing TPOH-containing neurons in this area. Bar = 100 μ m.